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## SYMPOSIUM

# Shorebirds' Seasonal Adjustments in Thermogenic Capacity Are Reflected by Changes in Body Mass: How Preprogrammed and Instantaneous Acclimation Work Together

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**Synopsis** Phenotypic flexibility in shorebirds has been studied mainly in the context of adjustments to migration and to quality of food; little is known on how birds adjust their phenotype to harsh winter conditions. We showed earlier that red knot (*Calidris canutus islandica*) can acclimate to cold by elevating body mass. This goes together with larger pectoral muscles, i.e., greater shivering machinery, and thus, better thermogenic capacity. Here, we present results of a yearlong experiment with indoor captive knots to determine whether this strategy is part of their natural seasonal phenotypic cycle. We maintained birds under three thermal regimes: constant cold (5°C), constant thermoneutrality (25°C) and natural seasonal variation between these extremes (9–22°C). Each month we measured variables related to the birds' endurance to cold and physiological maintenance [body mass, thickness of pectoral muscles, summit metabolic rate ( $M_{\text{sum}}$ ), food intake, gizzard size, basal metabolic rate (BMR)]. Birds from all treatments expressed synchronized and comparable variation in body mass in spite of thermal treatments, with a 17–18% increase between the warmest and coldest months of the year; which appeared regulated by an endogenous driver. In addition, birds living in the cold exhibited a 10% higher average body mass than did those maintained at thermoneutrality. Thickness of the pectoral muscle tracked changes in body mass in all treatments and likely contributed to greater capacity for shivering in heavier birds. Consequently,  $M_{\text{sum}}$  was 13% higher in cold-acclimated birds compared to those experiencing no thermoregulation costs. However, our data also suggest that part of maximal heat production comes from nonshivering processes. Birds facing cold conditions ate up to 25% more food than did birds under thermoneutral conditions, yet did not develop larger gizzards. Seasonal variation in BMR followed changes in body mass, probably reflecting changes in mass of metabolically active tissues. Just as cold-exposed birds, red knots in the variable treatment increased body mass in winter, thereby improving cold endurance. During summer, however, they maintained a lower body mass and thermogenic capacity compared to cold-exposed birds, similar to individuals kept at thermoneutrality. We conclude that red knots acclimate to seasonal variations in ambient temperature by modulating body mass, combining a preprogrammed increase in mass during winter with a capacity for fine-tuning body mass and thermogenic capacity to temperature variations.

## Introduction

Long-distance migratory shorebirds are capable of extraordinary feats of endurance exercise (Piersma 2011) and are faced with a great variety of ambient

conditions in the course of a year (Wiersma and Piersma 1994; Piersma and van Gils 2011). To support this way of life, they have evolved a high level of phenotypic flexibility, which is especially visible in

the morphological changes in response to fuelling, energy requirements and diet (Piersma 1997; Piersma and Drent 2003; van Gils et al. 2003). Indeed, components of the digestive system such as gizzard and intestine are known to grow during pre-flight fueling and regress just before departure (Piersma and Gill 1998; Piersma et al. 1999; Landys-Ciannelli et al. 2003), whereas flight muscles develop without power training (Dietz et al. 1999a) in the few days preceding take off (Piersma et al. 1999). Molluscivorous shorebirds such as the red knot (*Calidris canutus*) also adjust their digestive machinery in response to changes in the quality of their diet. They can double the size of their gizzard, which is highly correlated with the size of their intestine (Piersma et al. 2003), within a few days in response to a switch in diet toward bivalves containing a large amount of refractory shell material that needs crushing and transport through the digestive tract (Dekinga et al. 2001).

Although the physiological changes required to exploit a low-quality winter diet are now relatively well understood (e.g., Piersma et al. 1993, 2003; Dekinga et al. 2001; van Gils et al. 2003), thermoregulatory adjustments made in response to winter climatic conditions have received rather less attention. For shorebirds wintering in the tropics, high ambient temperatures can trigger a physiological response to avoid overheating. This may take the form of a decrease in metabolic rate that reduces endogenous heat production (Klaassen et al. 1990; Piersma et al. 1996; Kersten et al. 1998; Piersma 2002a). However, several species winter at northern latitudes and encounter relatively cold, windy and rainy weather (e.g., Summers et al. 1998). One of these species is our model, the *islandica* subspecies of the red knot, which winters on the mudflats of western Europe (Davidson and Wilson 1992; Quaintenne et al. 2011). For *islandica* knots wintering in the unpredictable climate of the Dutch Wadden Sea, predictive equations generated from taxidermic models calibrated against live animals (Wiersma and Piersma 1994) suggest that winter is the most energetically demanding time of the year in terms of maintenance metabolism (basal metabolic rate, BMR, plus the costs of thermoregulation). However, little is known on how these birds adjust their phenotype to winter conditions.

Earlier we showed in *islandica* knots that thermal acclimation under controlled conditions was associated with a 14–15% higher body mass, a 26% higher BMR, and a 13% higher summit metabolic rate ( $M_{\text{sum}}$ ) in captive birds acclimated to a low winter-like temperature (4°C) compared to birds

acclimated to thermoneutrality (26°C) (Vézina et al. 2006).  $M_{\text{sum}}$  is the maximal thermogenic capacity generated by a shivering individual under cold challenge (Swanson et al. 1996), and is correlated to cold endurance in birds (Swanson 2001; Swanson and Liknes 2006). Since the pectoral muscles are the largest avian muscle group (up to 19% of lean body mass in red knots, Piersma et al. 1996; Piersma and Dietz 2007), and because a knot increasing its mass also gains pectoral muscles and improve its thermogenic capacity (Lindström et al. 2000; Vézina et al. 2007), we concluded that captive knots had the capacity to acclimate to cold by modulating their body mass (Vézina et al. 2006). Observations of short-term changes in body mass in dunlins (*Calidris alpina*) facing winter climatic variations (Davidson et al. 1986a, 1986b; Kelly et al. 2002) also suggest that shorebirds acclimate to cold mainly by modulating body mass. Increasing body mass would improve the capacity for heat production, likely through gains in muscle size (Vézina et al. 2006, 2007, 2010). However, seasonal acclimatization by modulation of body mass in response to natural changes in ambient temperature remains to be demonstrated experimentally.

The short-term studies reported by Vézina et al. (2006, 2007) were part of a yearlong experiment in which seasonal changes in immune function (detailed by Buehler et al. 2008) and metabolic performance were uncoupled from physiological adjustments to ambient temperature under controlled conditions. Here, we report on phenotypic variation in body mass, metabolic performance and organ “machinery” in birds maintained under controlled conditions but experiencing naturally variable, outdoor temperatures. These are put in contrast with individuals facing constant winter-like or thermoneutral temperatures with the aim of determining whether shorebirds do acclimate to seasonal changes in temperature by modulating their body mass.

## Methods

### Experimental animals and diet

All birds used in this experiment were adult *islandica* red knots captured in 2004 in the Dutch Wadden Sea and brought into outdoor captivity (see Vézina et al. 2006 for details) at the shorebird facility of the Royal Netherlands Institute for Sea Research (NIOZ). Birds were transferred to indoor temperature-controlled aviaries in January 2005 and experimental groups were randomly formed as described by Vézina et al. (2006). We had five indoor aviaries at our disposal. Therefore, birds were divided into five

groups forming three different thermal treatments: (1) Two aviaries were ventilated with outside air, hereafter called “variable” treatment, and temperatures oscillated naturally between average monthly values of 9°C and 22°C throughout the year (maximum of 25.5°C on 24 June 2005; minimum of 4.6°C on 6 March 2006, Fig. 1A); (2) Two groups of birds were kept in heated aviaries where temperature was maintained within the thermoneutral zone at 21–26°C (mean  $25.0 \pm 1.4^\circ\text{C}$ ) (Fig. 1A), thereby forming the “thermoneutral” treatment (Wiersma and Piersma 1994; Piersma et al. 1995); and (3) The fifth group of birds, forming the “cold” treatment, was kept in one aviary maintained at temperatures comparable to the coldest months of winter in the Wadden Sea, with temperatures ranging between 4.1°C and 7.0°C (mean  $5.1 \pm 1.0^\circ\text{C}$ ) (Fig. 1A). Birds from all treatments experienced the same naturally changing photoperiod for this location with lights turning on and off gradually over a 20-min period during artificial “sunrise” and “sunset.”

The experiment began in February 2005. Groups were originally formed of five birds per aviary in thermoneutral and variable treatments and six birds initially in the cold treatment. Four additional birds were added by the end of the first month to bring all groups to the same size (six birds per aviary). Three birds died of unknown causes during the experiment. One bird died in April 2005 and one died in August 2005. These were replaced. One bird died in February 2006 and was not replaced as this was the last month of the experiment. All experimental groups had similar sex ratios and structural body size (Vézina et al. 2006). This experiment complied with the Dutch Law on Experimental Welfare and the animal welfare guidelines of the Royal Netherlands Academy of Art and Sciences.

### Monthly protocol

Every month, we repeated the same measurement protocol, which lasted 16 days. On the first day, all birds were captured for ultrasonographic measurements. Birds from all aviaries were weighed and kept in holding boxes until they were chosen in a random order. In ignorance of a bird’s experimental status, we noninvasively measured the thickness of their pectoral muscles and the width and height of their gizzard according to the methods of Dietz et al. (1999b) and Lindström et al. (2000), with the exception that each gizzard was measured twice and the average taken as the final value. Preliminary trials showed high repeatability of measurements (calculated according to Lessells and Boag (1987), pectoral

muscle  $r=0.97$ , gizzard height  $r=0.62$ , gizzard width  $r=0.65$ ). We missed observations for June, September and October due to scanner and/or operator being unavailable.

Our respirometry system limited measurements to two individuals per day. Therefore, we chose to work with two birds from the same aviary on each day of measurement to reduce disturbance in the other aviaries. The order in which specific birds were measured was randomly chosen and the same order was kept each month for the duration of the experiment. Since it took 15 days per month to measure every individual by respirometry, this insured that each bird was measured with exactly 1 month between each of its sessions. We began the respirometry protocol on the day of ultrasonic measurements (average time within month between ultrasonic and respirometry measurements per treatment; variable: 7.5 days, thermoneutral: 8 days, cold: 9 days). On that day, we removed the food tray from one of the aviaries at 17:00 (the aviaries had salt water continuously flowing on the floor and a small mud flat as well as a tray filled with fresh water for drinking). The next morning at 10:00, we captured two individuals from that specific aviary and food was given back to the other birds. These two birds were put in a holding box without food but with water until BMR measurement. It should be noted that red knots are used to fasting as their natural foraging activities are closely related to tidal cycles in which high tides prevent access to mudflats (van Gils et al. 2005, 2006). Removal of food, therefore, has little effect on the birds. Aviaries were visited like this once every 5 days during the 15-day sessions (time required to measure birds from all other aviaries before catching the next two birds from a specific aviary). Therefore, this protocol was repeated with two different birds from a different treatment every day until all individuals had their BMR and  $M_{\text{sum}}$  measured.

We measured BMR, defined here as the energy consumed by a resting postabsorptive knot measured at night and at thermoneutrality, using the setup and methods described by Vézina et al. (2006, 2007). Measurements began at 16:00 on the day the birds were taken from their aviaries and lasted until 9:00 the following morning. Birds were weighed before and after BMR sessions and average mass was used in the analysis. Within 30 min following the end of BMR measurement, the birds were put back into metabolic chambers for  $M_{\text{sum}}$  measurements. We used the sliding cold-exposure protocol of Swanson et al. (1996) described in detail by Vézina et al. (2006, 2007). Maximal thermogenic capacity



was measured in a helium–oxygen (helox) environment with temperatures ramping down by 5°C every 30 min, starting at –15°C.  $M_{\text{sum}}$  was attained when a decline in the chamber's temperature induced no further increase in  $\text{VO}_2$  or at the point preceding an hypothermic state (visible through a steady decline in  $\text{VO}_2$  for several minutes) (Swanson et al. 1996). Mean cloacal temperature following  $M_{\text{sum}}$  trials was  $35.8 \pm 2.5^\circ\text{C}$  confirming that the birds had reached an hypothermic state by the end of measurements; normal body temperature in red knots is  $42^\circ\text{C}$  (Vézina et al. 2007). As for BMR, body mass was measured before and after  $M_{\text{sum}}$  sessions and the average mass was used in the analysis. Air flow rate was set to 50 l/h during BMR and 205 l/h during  $M_{\text{sum}}$  measurements (metabolic chamber effective volume: 6.8 l). We used a sampling interval of 30 s and BMR and  $M_{\text{sum}}$  were obtained by extracting the lowest and highest mean values corresponding to 10 min of  $\text{VO}_2$  recorded during these respective trials (instantaneous correction applied on  $M_{\text{sum}}$  data; Bartholomew et al. 1981). Respiratory quotient was  $0.71 \pm 0.02$ , confirming that lipids were used as metabolic fuel and that birds were postabsorptive. Therefore,  $\text{VO}_2$  values were converted to Watts (W) using an energy equivalent of 20 kJ/l  $\text{O}_2$ . Oxygen and  $\text{CO}_2$  analyzers ( $\text{O}_2$ : Servomex Model 4100;  $\text{CO}_2$ : Servomex Model 1400, Servomex, Zoetermeer, The Netherlands) were calibrated daily with certified span gases and scrubbed air. Our validation tests showed that our system was accurate to 4% of real value (Vézina et al. 2006). Birds were actively molting during July and August (see Buehler et al. 2008). Because measuring  $M_{\text{sum}}$  in red knots requires exposing the birds to temperatures that can be below  $-25^\circ\text{C}$  (Vézina et al. 2007), we did not measure  $M_{\text{sum}}$  during these months to avoid health problems that could arise from frostbite of blood-filled feather pins.

### Food intake

Knots were fed with a diet composed solely of mud snails (*Hydrobia ulvae*) 2–4 mm long, often a considerable proportion of their natural winter diet (Dekinga and Piersma 1993, van Gils et al. 2003, Quaintenne et al. 2010). These tiny snails are consumed whole and are crushed in the gizzards to extract the flesh. We collected all *Hydrobia* during a one-time dredging expedition in the Wadden Sea to ensure constant quality of food throughout the experiment. Snails were stored frozen throughout the year and birds were fed in excess every day with a freshly thawed portion of snails offered in a

tray filled with salt water. We measured intake of food in all groups over a period of 24 h once a month by comparing the amount of food offered at 10:00 to the amount left over the following day at the same hour and then converting the values to ash-free dry mass (AFDM) consumed per bird after drying and hashing the samples (see Vézina et al. 2006 for details of the methods).

### Statistical analysis

Our experimental setup was based on a repeated-measure design in which birds were measured 13 times from February 2005 to 2006. Because different birds were assigned to different aviaries (and therefore formed different groups), our design implied nested effects (e.g., “individual” nested in “group” nested in “treatment”). Therefore we analyzed our data using a mixed GLM approach with variables “individual” and “group” treated as nested random variables. Since we were interested in seasonal changes in relation to experimental treatments, we included the factors “month,” “treatment” and the interaction term “treatment  $\times$  month” in all analyses.

Our analyses on BMR and  $M_{\text{sum}}$  used the same approach and were first conducted on whole values. We then investigated changes in mass-independent values by including body mass as a covariate in the models. We also included muscle thickness and gizzard size as covariates (gizzard width and height analyzed separately) to study their possible relationship with metabolic performance (BMR and  $M_{\text{sum}}$ ). In this specific case, we considered the effect of time between individual ultrasound and respirometry measurements to account for possible uncoupling of variations in organ size and metabolic rate. However, we found this effect to be nonsignificant in all cases and therefore excluded this variable from our analyses.

Our main objective was to study seasonal phenotypic changes in variable birds in comparison with phenotypes of individuals maintained in constant cold or at thermoneutrality throughout the year. As expected, the interaction term “treatment  $\times$  month” was significant in several cases (see Tables 1 and 2; Fig. 1). To refine our investigation, we therefore used a *post hoc* approach based on least-square means comparisons. Least-square means generated from the interaction term “treatment  $\times$  month” (shown in Fig. 1) were extracted for all variables. This provided an average value per month per treatment that was controlled for the repeated nature of our observations. The same approach was also used to extract

least-square means for monthly average ambient temperature and food intake (mixed GLM controlling for the random effect “group”). Using linear regressions, we then studied how monthly average ambient temperature was related to our variables (Fig. 2). This approach also allowed us to study the relationship between any given variable without problems that could arise from pseudoreplication. However, as ambient temperature changed with time within the variable treatment, temperature also reflected seasonality. Therefore, when examining phenotypic changes in birds exposed to the variable treatment, one is observing a combined effect of temperature and seasonality. To isolate the effect of ambient temperature, we compared phenotypes of birds exposed to cold with those of birds exposed to thermoneutral treatments.

It should be noted here that we excluded the month of May from all regression analyses as it corresponds to the peak of migratory fattening (see below). This study addresses seasonal effects and not those caused by migratory predisposition, which we covered elsewhere (Vézina et al. 2007).

We confirmed normality of residuals and homogeneity of variances for all analyses. Data are presented as mean  $\pm$  SE.

## Results

Red knots showed considerable phenotypic flexibility. Indeed, all traits changed significantly in the course of the year (significant effect of month in all cases) (Tables 1 and 2), but monthly variation was complex and dependent on treatment for several traits (significant interaction term treatment  $\times$  month) (Fig. 1). Although ambient temperature had a clear effect on phenotype (Fig. 2), seasonal variation within treatment (see below) prevented us from detecting a significant effect of treatment in most cases (Tables 1 and 2).

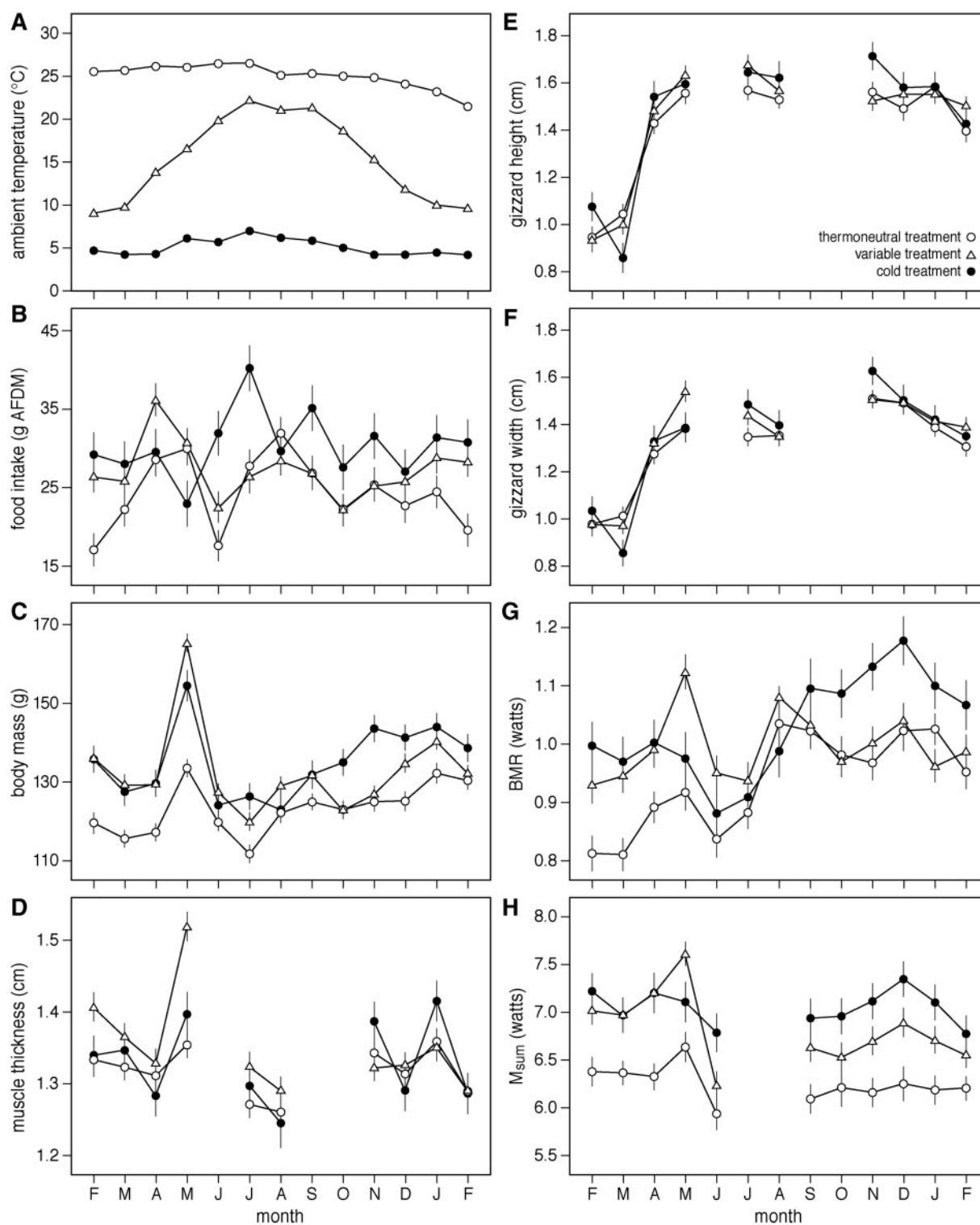
### Food intake

Red knots facing the cold environment had extra costs of thermoregulation and thus ate more food than did birds facing variable or thermoneutral conditions (significant treatment effect) (Table 1). Indeed, the average amount of food consumed by cold-acclimated birds over the whole year ( $30.4 \pm 0.7$  g/d/bird AFDM) was 11.8 and 25.1% higher than that consumed by variable and thermoneutral birds, respectively (variable:  $27.2 \pm 0.5$  g/d/bird AFDM; thermoneutral:  $24.3 \pm 0.5$  g/d/bird AFDM, significant difference between cold and

thermoneutral treatments with variable not differing from either of those; *post hoc* Tukey test). The treatment effect on food intake was also complicated by a significant interaction term treatment  $\times$  month (Table 1 and Fig. 1B). However, studying the monthly least-square means in relation to average ambient temperature revealed a clearer picture (Fig. 2A). Cold-acclimated birds ate more food than did those maintained at thermoneutrality, while individuals facing a variable environment tended to change their food consumption between these two extremes in relation to ambient temperature (Fig. 2A,  $r^2 = 0.33$ ,  $n = 36$ ,  $P < 0.001$ ).

### Body mass

Birds from all treatments showed a similar pattern of variation in body mass over the year (month effect, Table 1), but the effect of thermal treatment varied through time (significant interaction term treatment  $\times$  month) (Table 1 and Fig. 1C). The month of May was marked by a clear increase in body mass in birds from all treatments, consistent with an endogenous annual clock orchestrating migratory fueling (Cadée et al. 1996; Piersma 2002b; Reneerkens et al. 2007; Piersma et al. 2008). The circannual cycle in body mass was also visible during the rest of the year, as birds from all treatments increased their body mass during the winter months (Fig. 1C). In fact, comparing the lowest and highest monthly least-square means per treatment over the whole year (excluding May), revealed that all birds changed their body mass by surprisingly similar amounts between summer and winter regardless of their thermal environment (cold: 17.1% increase between August and January; variable: 17.2% between July and January; thermoneutral: 18.2% between July and January) (Fig. 1C). This seasonal effect generated an overlap in the data among the treatments (e.g., see data spread within treatment in Fig. 2B) which prevented us from detecting a significant overall treatment effect on body mass (Table 1). Nevertheless, visual inspection of the data (Fig. 2B) clearly showed that cold-acclimated birds maintained a higher average body mass (9.7% difference) than birds kept at thermoneutrality (least-square means calculated from mixed model; cold:  $135.2 \pm 6.0$  g; variable:  $132.8 \pm 3.6$  g; thermoneutral:  $123.3 \pm 3.6$  g). Therefore, our data suggest that red knots not only have a clock-related driver to their yearly variation in body mass, but also respond to the lowering of temperature by gaining weight. Accordingly, the body mass phenotype observed in variable birds was approaching that of the cold-acclimated birds during



**Fig. 1** Seasonal variation in relation to thermal treatment in (A) ambient temperature, (B) food intake, (C) body mass, (D) muscle thickness, (E) gizzard height, (F) gizzard width, (G) BMR, and (H)  $M_{sum}$ . All variables are least-square means extracted from a mixed general linear model testing for the effects of month, treatment, group and the interaction term treatment  $\times$  month. Values presented in (C to H) also control for the effect of individual bird. See text for details. Error bars are smaller than symbols in (A). AFDM=ash free dry mass.

**Table 1** Mixed GLM testing for the effect of month and treatment on food intake, body mass, muscle thickness, and gizzard size while controlling for the random effect of group and individual

Dependent variables																	
Food intake			Body mass			Muscle thickness			Gizzard height			Gizzard width					
Independent variables	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	P
Month	12, 24	3.9	<0.005	12, 314	25.4	<0.0001	9, 232	12.3	<0.0001	9, 235	65.2	<0.0001	9, 234	47.3	<0.0001	9, 234	<0.0001
Treatment	2, 2	21.7	<0.05	2, 1.9	2.3	0.3	2, 1.9	0.2	0.9	2, 3.9	6.6	0.06	2, 3.1	4.0	0.1	2, 3.1	0.1
Group (treatment)	2, 24	0.9	0.4	2, 26.6	2.8	0.08	2, 26.2	9.6	<0.001	2, 21.4	0.5	0.6	2, 22.1	0.6	0.5	2, 22.1	0.5
Individual (group [treatment])	–	–	–	27, 314	11.5	<0.0001	27, 232	6.3	<0.0001	27, 235	0.7	0.9	27, 234	0.9	0.7	27, 234	0.7
Treatment × month	24, 24	2.4	<0.05	24, 314	3.9	<0.0001	18, 232	2.7	<0.0005	18, 235	1.3	0.2	18, 234	1.1	0.3	18, 234	0.3
Total R <sup>2</sup>		0.87			0.76			0.69			0.75						0.70

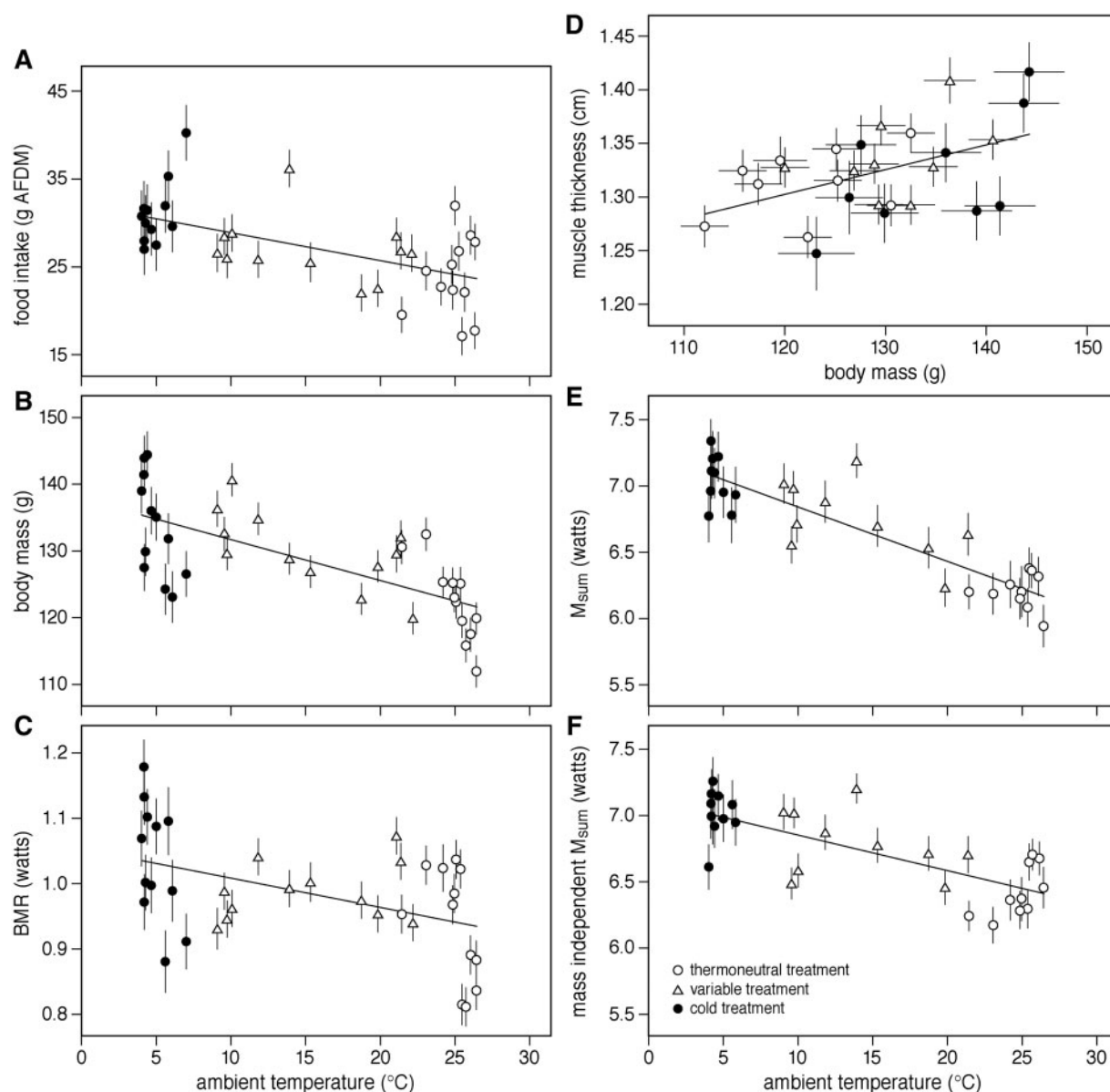
Note. P values in bold are referred to in the text.

**Table 2** Mixed GLM testing for the effect of month and treatment on basal and summit metabolic rate while controlling for the random effect of group and individual

Dependent variables														
BMR			BMR			M <sub>sum</sub>			M <sub>sum</sub>					
Independent variables	df	F	P	df	F	P	df	F	P	df	F	P		
Month	12, 307	9.3	<0.0001	12, 306	7.8	<0.0001	10, 212	5.4	<0.0001	10, 211	4.4	<0.0001		
Treatment	2, 18	5.2	0.2	2, 2.1	12.2	0.07	2, 1.9	1.7	0.4	2, 1.9	1.5	0.4		
Group (treatment)	2, 26.1	0.9	0.4	2, 26.3	0.2	0.9	2, 27.8	4.6	<0.05	2, 28.0	2.7	0.09		
Individual (group [treatment])	27, 307	5.5	<0.0001	27, 306	3.4	<0.0001	27, 212	8.1	<0.0001	27, 211	8.7	<0.0001		
Treatment × month	24, 307	2.7	<0.0001	24, 306	1.4	0.1	20, 212	1.1	0.4	20, 211	0.6	0.9		
Body mass	–	–	–	1, 306	67.3	<0.0001	–	–	–	1, 211	61.6	<0.0001		
Total R <sup>2</sup>		0.58			0.66			0.74			0.80			

Note. Analyses are presented both without and with body mass as a covariate. P values in bold are referred to in the text.





**Fig. 2** Relationship between least-square mean (LSM) ambient temperature and LSM (A) food intake, (B) body mass, (C) BMR, (E)  $M_{sum}$ , and (F) mass-independent  $M_{sum}$ . (D) shows the relationship between LSM body mass and LSM muscle thickness. All LSM values were extracted from mixed general linear models and are the same as shown in Figure 1 except for the month of May, the period of migratory predisposition, which was excluded from analyses. See text for details. Error bars for ambient temperature are smaller than symbols. AFDM=ash free dry mass.

the winter months and was similar to the thermoneutral phenotype during the summer period ( $r^2=0.49$ ,  $n=36$ ,  $P<0.0001$ ) (Fig. 2B).

#### Muscle thickness and gizzard size

Thickness of the pectoral muscles also varied throughout the year and was dependent on the relationship between thermal treatment and month (significant month effect and treatment  $\times$  month interaction term.) (Table 1 and Fig. 1D). However, the effects of treatment and season were not as clear (no significant relationship between least-square

mean muscle thickness and ambient temperature;  $P=0.3$ ). Comparing least-square means for muscle thickness and body mass nevertheless confirmed that muscle size tracked body mass throughout the year ( $r^2=0.22$ ,  $n=27$ ;  $P<0.05$ ) (Fig. 2D). Therefore, when birds were heavy, they also had larger muscles, whether they were acclimated to cold or not.

Birds from all treatments showed seasonal variation in gizzard height and width (significant month effect) (Table 1), but this effect was generated by an increase in gizzard size in April to reach values that remained elevated for the remainder of the

experiment (Fig. 1E and F). Although a change in the quality of diet by the third month could have generated such an effect, our procedures were such that this is unlikely. Indeed, the proportion of ash in the food (i.e., the percent of shell per unit mass) remained constant throughout the experiment (at 78%, no month-effect) and least-square mean analysis showed that gizzard size was not related to either the total amount of dry food (including shell) or mass of ash ingested (shell only). Although cold-acclimated birds consumed more digestible material than did individuals facing variable and thermoneutral conditions, this treatment effect on intake of food did not translate into significantly larger gizzards in birds living in the cold. At best, there was a nonsignificant trend for gizzard height being 4.2% higher in cold-acclimated birds compared to individuals living at thermoneutrality (treatment effect:  $P=0.06$ ) (Table 1), but this was not visible in gizzard width ( $P=0.1$ ). The analysis on least-square means revealed no significant relationship between gizzard size and ambient temperature ( $P=0.9$  in both width and height; removing the first two months of the experiment did not change this result;  $P=0.2$ – $0.3$ ).

### Metabolic performance

Whole BMR varied throughout the year (significant month effect) (Table 2) but was also dependent on thermal treatment (significant treatment  $\times$  month interaction term) (Table 2 and Fig. 1G). Although whole BMR was on average 9.6% higher in cold-acclimated birds ( $1.03 \pm 0.03$  W) than in individuals maintained at thermoneutrality [ $0.94 \pm 0.02$  W (see Fig. 2C); relationship with least-square mean ambient temperature  $r^2=0.21$ ,  $n=36$ ,  $P<0.005$ ], we could not detect a significant treatment effect on BMR in our mixed model (Table 2). In fact, seasonal variation in BMR mirrored changes in body mass (compare panels c and g in Fig. 1). Including body mass as a covariate resulted in a nonsignificant interaction term treatment  $\times$  month (Table 2) and a trend for a 5.2% higher mass-independent BMR in cold versus thermoneutral individuals ( $P=0.07$ ; Table 2). Least-square means extracted from this model (i.e. mass independent BMR) were not related to least-square mean ambient temperature ( $P=0.1$ ).

Including organ size in the analysis on BMR revealed a positive relationship between BMR and muscle thickness ( $F_{1,226}=6.3$ ;  $P<0.05$ ), but this effect disappeared when body mass was added as a covariate ( $P=0.5$ ; removing the nonsignificant interaction term did not change this result).

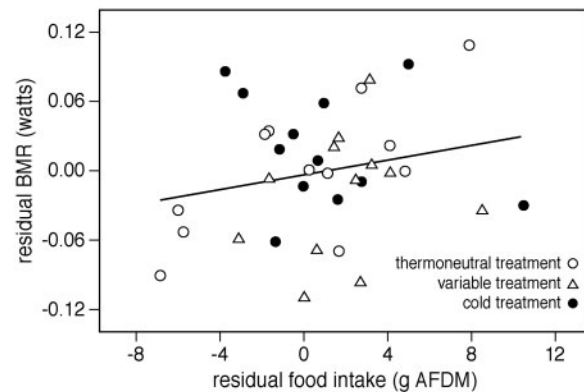


Fig. 3 Relationship between residual LSM food intake (effect of LSM ambient temperature removed) and residual LSM BMR (effect of LSM body mass removed). See text for details. AFDM=ash free dry mass.

Both measures of gizzard size were not related to BMR of the whole animal ( $P=0.9$  in both cases) or when including body mass as a covariate ( $P>0.6$  in both cases; removing the nonsignificant interaction term did not change this result). However, least-square mean analysis revealed that residual BMR (controlling for body mass) was significantly correlated with residual monthly food intake (controlling for the effect of monthly ambient temperature) ( $r^2=0.11$ ,  $n=36$ ,  $P<0.05$ ) (Fig. 3). Therefore, for a given body mass and season, birds that consumed more food had a higher BMR.

Like the other parameters recorded in this experiment, maximal thermogenic capacity changed throughout the year (significant month effect) (Table 2). However, birds from all treatments expressed a relatively parallel variation in  $M_{\text{sum}}$  (no significant treatment  $\times$  month interaction term) (Fig. 1H). Although we could not detect a significant treatment effect on  $M_{\text{sum}}$  in a mixed model (Table 2), the analysis of least-square means revealed a clear pattern in which cold-acclimated birds nevertheless showed a 12.8% higher average  $M_{\text{sum}}$  ( $7.1 \pm 0.4$  W) compared to individuals at thermoneutrality ( $6.3 \pm 0.3$  W), while birds facing variable conditions adjusted their thermogenic performance between these extremes according to seasonal changes in ambient temperature ( $r^2=0.78$ ,  $n=30$ ;  $P<0.0001$ ) (Fig. 2E). Thermogenic capacity was not affected by gizzard size ( $P>0.6$  for both measures; removing the nonsignificant interaction term did not change this result) but was positively related to thickness of the pectoral muscle ( $F_{1,168}=4.0$ ,  $P<0.05$ , nonsignificant interaction removed). Like BMR, however,  $M_{\text{sum}}$  was significantly affected by body mass (Table 2) and controlling for body mass

removed the effect of muscle thickness on  $M_{\text{sum}}$  (muscle thickness effect,  $P=0.2$ ). This is not surprising, since body mass includes muscle mass. Missing monthly values for  $M_{\text{sum}}$  and muscle thickness could also have reduced our chances of detecting a mass-independent effect of muscle size on thermogenic capacity.

Interestingly, controlling for body mass did not eliminate the relationship between least-square mean  $M_{\text{sum}}$  and least-square mean ambient temperature ( $r^2=0.57$ ,  $n=30$ ,  $P<0.0001$ ) (Fig. 2F). Therefore, for a given body mass, cold-acclimated birds not only expressed a higher thermogenic capacity than that of birds maintained at thermoneutrality (average values for cold:  $7.0 \pm 0.3$  W; thermoneutral:  $6.4 \pm 0.2$  W), but individuals kept in the variable environment also had a higher mass-independent  $M_{\text{sum}}$  during winter months. Consequently, birds facing cold conditions gained thermogenic capacity in association with an increase in body mass and muscle size but also increased their level of mass-independent heat production.

Least-square means analysis exposed no relationship between  $M_{\text{sum}}$  and food intake ( $P>0.2$ ) but revealed a significant relationship between average whole BMR and average whole  $M_{\text{sum}}$  ( $r^2=0.26$ ,  $n=30$ ;  $P<0.005$ ). However, this was driven by an underlying effect of body mass. Performing the analysis on mass-corrected data (extracted from mixed models) resulted in no significant relationship ( $P=0.4$ ).

## Discussion

### Phenotypic variation in body mass, muscle thickness, and thermogenic capacity

We followed mass and thermogenic capacity in captive red knots facing naturally variable ambient temperatures over a year and compared them with measures of birds kept in constant winter-like cold and thermoneutral conditions under the natural photoperiod for the area. As observed previously (Vézina et al. 2006), birds kept in the cold ate more food than did birds kept at thermoneutrality; they also maintained a higher body mass (Fig. 2A and B). Although treatment-effect alone was not significant in a mixed model, the average difference in body mass between these extreme treatments was nevertheless 10% over the whole year, a value slightly lower than the 14–15% we reported earlier for the same birds (Vézina et al. 2006).  $M_{\text{sum}}$  on the other hand, was on average 13% higher in cold-acclimated birds than in individuals maintained at thermoneutrality (Fig. 2E), which corresponds to the difference

we observed before (Vézina et al. 2006). Individuals facing naturally variable conditions changed their phenotype throughout the year according to seasonal variation in ambient temperature (Fig. 2). Body mass and thermogenic capacity were higher during the winter months, approaching the cold-acclimated phenotype, and were lower in summer months, then being more comparable to the thermoneutral phenotype. These observations are therefore consistent with our working hypothesis. Red knots do acclimate to seasonal variations in temperature by modulation of body mass. However, the phenomenon appears to be more complex than a simple response to ambient temperature.

Considering the complete year, we also observed seasonal patterns of variation in body mass that were similar in all thermal treatments. First, there was the well-known peak of mass during May, which coincides with the fuelling episode associated with spring migration (Cadée et al. 1996; Piersma 2002b; Reneerkens et al. 2007). This phenomenon is routinely observed in captive red knots (e.g., Piersma et al. 1995). It is maintained by an endogenous circannual clock and fine-tuned by adjustments to the photoperiod (Cadée et al. 1996; Piersma 2002b; Reneerkens et al. 2007; Piersma et al. 2008). Second, and perhaps more interesting in the context of this study, we observed an increase in body mass in all treatments during the winter months (Fig. 1C), a phenomenon also known in free-living *islandica* knots (see Fig. 33 in Piersma 1994). In fact, all birds expressed a remarkably similar seasonal variation in average body mass, increasing by 17–18% between the warmest (June–July) and coldest (January) months of the year. As for the migratory fuelling episode, this seasonal pattern was observed in all treatments and was therefore independent of temperature. Here again, this is consistent with an endogenous control of body mass by an internal clock or calendar, which is likely fine-tuned by photoperiod (Cadée et al. 1996; Piersma 2002b; Piersma et al. 2008). Consequently, our observations strongly suggest that seasonal changes in body mass, and correlated effects on thermogenic capacity and cold endurance, are driven by a combination of preprogrammed seasonal variation and a temperature-dependent response. Red knots endogenously adjust their phenotype in winter by increasing their body mass, but still maintain a capacity for fine-tuning their response to local variation in ambient temperature by maintaining extra capacity (10% in this case) for modulation of body mass.

Red knots wintering on European mudflats face unpredictable weather conditions characterized by

low temperature, high wind and heavy precipitation (Wiersma and Piersma 1994). Therefore, part of the preprogrammed increase in body mass in winter may be reflecting enlarged nutritional stores supporting seasonally high energy demands and unpredictable fasting (e.g., Vézina et al. 2009a). However, it is now well established that variations in body mass in shorebirds are tracked by adjustments in size of the pectoral muscle (Lindström et al. 2000; Dietz et al. 2007; Vézina et al. 2007; this study). Although the primary function of increasing the size of pectoral muscles may be the maintenance of flight capacity and maneuverability (Lindström et al. 2000; Dietz et al. 2007), a heavier body mass and larger muscles also result in a better capacity to produce heat (Vézina et al. 2007). In the context of seasonal acclimatization this clearly is an advantage.

As one would expect, based on this reasoning, we found a significant effect of the size of pectoral muscles on  $M_{\text{sum}}$ . Not surprisingly, this effect disappeared when including body mass (which encompasses the mass of all muscles) as a covariate in the mixed model. What was unexpected, however, was the finding of a significant relationship between  $M_{\text{sum}}$ , corrected for body mass, and ambient temperature across treatments and seasons (Fig. 2F). This finding indicates that birds facing a cold environment, whether they were from the cold or variable treatments, not only improved their endurance to cold by increasing their body mass but also improved their capacity for heat production *per unit* body mass. This was not caused by pectoral muscles being disproportionately large relative to body mass in birds living in the cold since the relationship between muscle thickness and body mass did not differ between treatments (Fig. 2D). Therefore, although the size of muscles such as *pectoralis* may play an important role in an individual's capacity to produce heat under cold stress (Hohtola 2004; Vézina et al. 2006, 2007, 2010; Swanson et al. 2009; Swanson 2010), there is likely another layer of heat-production capacity above that resulting from larger muscles, and since this is mass-independent thermogenic capacity, it must take place at the tissue level.

Maximal heat production in knots may, therefore, involve both a shivering and a nonshivering component. Elevation of metabolic intensity (i.e., heat production per unit tissue mass; McKechnie 2008; Swanson 2010) could be involved in nonshivering heat production and may result from adjustments in tissue mitochondrial density, upregulation of avian mitochondrial uncoupling proteins (avUCP, reviewed in Dridi et al. 2004) or increased activity

of oxidative enzymes such as citrate synthase or cytochrome C oxidase (reviewed in Swanson 2010, but see Weber and Piersma 1996; Selman and Evans 2005). Recent findings demonstrated that these processes might be under endocrine control (Dridi et al. 2004; Liu et al. 2006) and therefore suggest that short-term flexible changes in metabolic intensity could be adjusted to local thermal constraints, and possibly supplement shivering heat production. Triiodothyronine ( $T_3$ ), a thyroid hormone, could be regulating this process (Dridi et al. 2004; Liu 2006).  $T_3$  is known for its stimulatory effect on metabolic intensity (Carter et al. 1971; Deaton et al. 1997; Hulbert 2000; Short et al. 2001; Liu et al. 2006; Zheng et al. 2008), and its plasma titers increase in birds and mammals exposed to cold conditions (Bobek et al. 1980; Brigmon et al. 1992; Hulbert 2000; Jenni-Eiermann et al. 2002; Cherel et al. 2004; Duriez et al. 2004).  $T_3$  is also positively correlated with BMR in several species of birds (Bobek et al. 1977; Chastel et al. 2003; Duriez et al. 2004; Ronning et al. 2008) including red knots (Vézina et al. 2009b). Although  $T_3$  has been extensively studied for its role in the physiological response of vertebrates to cold (Hulbert 2000), we are unaware of any study investigating the regulation of *maximal* thermogenic capacity by thyroid hormones. As  $M_{\text{sum}}$  reflects cold endurance (Swanson 2001; Swanson and Liknes 2006), endocrine regulation of nonshivering heat production could provide a capacity for the birds to respond quickly to rapid climatic variations without requiring the synthesis of new shivering tissues. More experimental studies are needed to test this hypothesis.

### Phenotypic variation in basal metabolic rate

Cold acclimation or acclimatization in birds is often associated with an increase in BMR (reviewed by McKechnie 2008; Swanson 2010; McKechnie and Swanson 2010). Accordingly, we previously observed a 16% higher whole BMR and a 14% higher mass-independent BMR in cold-acclimated knots compared to individuals kept at thermoneutrality (Vézina et al. 2006). Such a cold-induced elevation of BMR may be driven primarily by enlarged metabolically active organs, such as the liver, kidney, and intestine (first suggested by Kersten and Piersma 1987, and see e.g., Piersma et al. 1996; Williams and Tieleman 2000; Tieleman et al. 2003; Cavieres and Sabat 2008; Zheng et al. 2008; Barcelo et al. 2009; Maldonado et al. 2009). However, BMR reflects the cumulative energy consumption of all physiological systems composing an animal at rest



(Piersma 2002a, McKechnie 2008, McKechnie and Swanson 2010) and, because these components are often highly flexible (Piersma and Lindström 1997), efforts to identify the specific system causing BMR to change at a particular time can be complicated and context-specific (Piersma et al. 2004; Vézina and Williams 2005; Vézina et al. 2009c; Swanson 2010).

We found that the effect of thermal treatment on BMR changed throughout the year (Fig. 1G). Overall, cold-acclimated birds maintained a 10% higher BMR compared with individuals kept at thermoneutrality (Fig. 2C), but the treatment effect *per se* was not significant. In fact, seasonal variation in BMR within treatments mainly resulted from changes in the birds' mass, despite a nonsignificant trend for higher metabolic intensity in cold-acclimated birds. Although individuals experiencing constant cold conditions consumed 25% more food on average than did those facing no thermoregulatory costs, gizzard size did not differ between treatments and was not related to BMR. Therefore, our data confirm our earlier observations. Knots living in the cold consumed more *Hydrobia* snails, but did not develop larger digestive organs, possibly by adopting a strategy of feeding continuously on small amounts of food (see Vézina et al. 2006). Nevertheless, for a given temperature across treatments and seasons, birds consuming more snails still had a higher mass-independent BMR (Fig. 3). These birds might have been able to avoid maintaining large gizzards and intestines but nevertheless had to develop or upregulate other organs and functions leading to a higher BMR.

Taken together, our findings suggest that an increase in BMR is not a prerequisite for cold-acclimation in red knots. Rather, it appears that BMR simply tracks changes in the amount and activity of metabolically active tissues in this species (Piersma et al. 1996; Piersma 2002a; Piersma and van Gils 2011). These findings are consistent with the lack of difference between summer and winter BMR reported earlier in outdoor captive red knots (Vézina et al. 2009b). Furthermore, mass-independent BMR and  $M_{\text{sum}}$  were not correlated. Therefore, we argue here again (see Vézina et al. 2006), that these variables reflect different sets of physiological components that are flexibly adjusted under seasonal acclimatization to cold (see also Swanson 2010).

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